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APPENDIX TO PRELIMINARY AMENDMENT OF JUNE 7, 2002

Version with Markings to Show Changes Made

Amendments to the Specification

On page 13, the paragraph beginning on line 19:

The expression plasmids can also take the form of shuttle vectors between a bacterial host such as Escherichia coli and yeasts; in this case an origin of replication and a selectable marker that function in the bacterial host would be required. It is also possible to position restriction sites which are unique on the expression vector such that they flank the bacterial sequences. This allows the bacterial sequences to be eliminated by restriction cleavage, and the vector to be religated prior to transformation of yeast, and this can result in a higher plasmid copy number and enhanced plasmid stability. Certain restriction sites such as 5'-GGCCNNNNNGGCC-3' (SfiI) (SEQ ID NO. 1) or 5'-GCGGCCGC-3' (NotI) are particularly convenient since they are very rare in yeasts and are generally absent from an expression plasmid.

On page 14, the paragraph beginning on line 19:

Figure 1: Oligodeoxynucleotides (represented by SEQ ID NOs. 22 and 23) used to generate the MstII and HindIII-SmaI restriction sites, situated respectively upstream and downstream of the V1V2 domains of the CD4 molecule.

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On page 14, the paragraph beginning on line 22:

Figure 2: Nucleotide sequence of the MstII-SmaI restriction fragment including the V1 and V2 domains of the CD4 receptor of the HIV-1 virus is represented by SEQ ID NO. 24. The recognition sites for MstII, HindIII and SmaI are underlined.

On page 14, the paragraph beginning on line 31:

Figure 8: Nucleotide sequence (SEQ ID NO. 25) of restriction fragment HindIII coding for the protein fusion prepro-HSA-V1V2 (SEQ ID NO. 26). Black arrows indicate the end of the "pre" and "pro" regions of HSA. The MstII site is underlined.

On page 18, the paragraph beginning on line 4:

Figure 34: Introduction of the "Leucine Zipper" of c-jun (BglIII-AhaII fragment) in a hybrid protein HSA-CD4. The nucleotide sequence of the BglII-AhaII fragment is represented by SEQ ID NO. 27 and the corresponding amino acid sequence is represented by SEQ ID NO. 28.

On page 18, the paragraph beginning on line 25:

Figure 36: Panel a: representation of several HindIII (-25)-MstII restriction fragments corresponding to deletions in HSA. Amino add position (numbered according to mature HSA) is indicated in parentheses.

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Panel b: detail of the position of the MstII site in one of the deletants (clone YP63, linker insertion at amino acid 495). The mature HSA nucleotide sequence surrounding amino acid 495 is depicted as SEQ ID NO. 29 and its corresponding amino acid sequence is depicted as SEQ ID NO. 30. The nucleotide sequence of clone YP63 surrounding amino acid 495 is depicted as SEQ ID NO. 31 and its corresponding amino acid sequence is depicted as SEQ ID NO. 32.

On page 18, the paragraph beginning on line 31:

Figure 37: Examples of the hinge regions between the HSA and CD4 moieties. The amino add pairs that are potential targets of endoproteases involved in the secretory pathway are boxed. Panel 1: hinge region of protein HSA_{585} -CD4 (SEQ ID NO. 33). Panel 2: hinge region of HSA_{Bal31} -CD4 proteins (SEQ ID NO. 34) obtained by Bal31 deletion of the C-terminal portion of HSA (in this representation the Lys--Lys pairs situated at the beginning of the CD4 moiety have been modified by site-directed mutagenesis as exemplified in E.13.2.). Panel 3: hinge region obtained by insertion of a polypeptide (shown here a fragment of troponin C) (SEQ ID NO. 35), obtained after site-directed mutagenesis using oligodeoxynucleotide Sq1445. Panel 4: general structure of the hinge region between the HSA and CD4 moieties.

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On page 19, the paragraph beginning on line 11:

Figure 38: Panel 1: structure of the in-frame fusion between the prepro region of HSA and the CD4 receptor, present notably in expression plasmids pYG373B, pYG380B, pYG381B and pYG560. Panel 1a: the amino acid pairs in SEQ ID NO. 36 that are potential targets of endoproteases involved in the secretory pathway are boxed. Panel 1b: These amino acid pairs in SEQ ID NO. 37 can be modified by mutating the second lysine of each pair such that the pair is no longer a target for such endoproteases. Panel 2: Examples of hinge regions between the CD4 and HSA moieties present notably in hybrid proteins V1-HSA (panel 2a) (SEQ ID NO. 38) or V1V2-HSA (panels 2b and 2c) (SEQ ID NOs. 39 and 40, respectively). Panel 3: general structure of the hinge region between the CD4 and HSA moieties (SEQ ID NO. 41).

On page 22, the paragraph beginning on line 6:

The NdeI site of plasmid pXL322 (Latta M. et al., Bio/Technology 5 (1987) 1309-1314) including the ATG translation initiation codon of prepro-HSA was changed to a HindIII site by oligodeoxynucleotide-directed mutagenesis using the following strategy: the HindIII-BglI fragment of pXL322 containing the 5' extremity of the prepro-HSA gene was cloned into vector M13mp18 and mutagenized with oligodeoxynucleotide 5'-ATCTAAGGAAATACAAGCTT-**ATG**AAGTGGGT-3' (SEQ ID NO. 2) (the HindIII site is underlined and the ATG codon of prepro-HSA is shown in bold type); the phage

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obtained after this mutagenesis step is plasmid pXL855 whose restriction map is shown in Figure 3. After verification of the nucleotide sequence, the complete coding sequence for prepro-HSA was reconstituted by ligation of the HindIII-PvuII fragment derived from the replicative form of the mutagenized phage and coding for the N-terminal region of prepro-HSA, with the PvuII-HindIII fragment of plasmid pXL322 containing the C-terminal of HSA, thereby generating a HindIII fragment coding the entire prepro-HSA gene. This *Hind*III fragment, which also contains a 61 bp nontranslated region at its 3' extremity, was cloned into the corresponding site of plasmid pUC8 to generate plasmid pXL869 (Figure 3).

On page 22, the paragraph beginning on line 27:

Plasmid pYG12 contains a 1.9 kb SalI-BamHI restriction fragment carrying the promoter region (1.5 kb) and terminator region (0.4 kb) of the PGK gene of S. cerevisiae (Figure 4). This fragment is derived from a genomic HindIII fragment (Mellor J. et al., Gene 24 (1983) 1-14) from which a 1.2 kb fragment corresponding to the structural gene has been deleted, comprising a region between the ATG translation initiation codon and the BglII site situated 30 codons upstream of the TAA translation termination codon. The HindIII sites flanking the 1.9 kb fragment were then destroyed using synthetic oligodeoxynucleotides and replaced by a SalI and a BamHI site respectively upstream of the promoter region and downstream of the transcription terminator of the PGK gene. A unique HindIII site was then introduced by site-directed mutagenesis at the junction of the promoter and terminator regions; the sequence flanking this unique HindIII site (shown in bold letters) is as follows:

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5'-TAAAAACAAAAGATCCCC**AAGCTT**GGGGATCTCCCATGTCTCTACT-3' <u>(SEQ ID NO. 3)</u>

On page 23, the paragraph beginning on line 8:

Plasmid pYG208 is an intermediate construction generated by insertion of the synthetic adaptor <u>Bam</u>HI/<u>Sal</u>I/<u>Bam</u>HI (5'-GATCCGTCGACG-3') <u>(SEQ ID NO. 4)</u> into the unique <u>Bam</u>HI site of plasmid pYG12; plasmid pYG208 thereby allows the removal of the promoter and terminator of the <u>PGK</u> gene of <u>S. cerevisiae</u> in the form of a <u>Sal</u>I restriction fragment (Figure 4).

On page 23, the paragraph beginning on line 30:

In addition, the utilization of oligodeoxynucleotides Sq451 and Sq452 which form a <u>Hind</u>III-<u>BstE</u>II adaptor is described in the same document and permits the generation of a <u>Hind</u>III restriction fragment composed of the 21 nucleotides preceding the ATG initiator codon of the <u>PGK</u> gene, followed by the gene coding for prepro-HSA. The nucleotide sequence preceding the ATG codon of such an expression cassette is as follows (the nucleotide sequence present in the <u>PGK</u> promoter of <u>S. cerevisiae</u> is underlined):

5'-AAGC<u>TTTACAACAAATATAAAAACA**ATG**</u>-3' <u>(SEQ ID NO. 5)</u>.

On page 27, the paragraph beginning on line 17:

5'-TTACATTATTAATTTAAAA <u>ATG</u> <u>GAT</u> <u>TTC</u> <u>AAA</u> <u>GAT</u> <u>AAG</u> <u>GCT</u> <u>TTA</u> <u>AAT</u> <u>GAT</u> <u>CTA</u> *AGG CCG CGA TTA AAT TCC AAC* . . . -3' <u>(SEQ ID NO. 6)</u>

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On page 28, the paragraph beginning on line 23:

5'-CCTTAGGCTTA**TAA**CATCACATTTAAAAGCATCTCAGCCTA

CCATGAGAATAAGAGAAAGAAAATGAAGATCAAAAGCTT-3' (SEQ ID NO. 7)

On page 36, the paragraph at line 3:

5'-GTGTTTCGTCGA**GACG**CCCACAAGAGTGAGG-3' (SEQ ID NO. 8).

On page 36, the paragraph at line 11:

5'-GGTGTGTTT**CGT**AGATCTGCACACAAGAGTGAGG-3' (SEQ ID NO. 9)

On page 36, the paragraph at line 31:

5'-*CCAGGGGTGTGTTTCGTCGA***AAGAAAGTGGTGCTGGGC**-3' (SEQ ID NO. 10)

On page 37, the paragraph at line 11:

5'-CCAACTCTGACACC**GACGCC**CACCTGCTTCAGG-3' (SEQ ID NO. 11).

On page 37, the paragraph beginning on line 22:

The introduction of an AhaII site at the end of the V2 domain of the CD4 receptor was accomplished by site-directed mutagenesis using oligodeoxynucleotide Sq1186 and plasmid pYG368, to generate plasmid pYG363 (Figure 28). The sequence of oligodeoxynucleotide Sq1186 is (the AhaII site is shown in bold type): 5'-GCTAGCTTTC**GACGCC**GGGGGAATTCG-3' (SEQ ID NO. 12). Plasmid pYG363 therefore carries a HindIII-AhaII fragment composed of the 21 nucleotides preceding the

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ATG codon of the PGK gene of S. cerevisiae followed by the coding sequence for the HSA prepro region fused to the V1V2 domains of the CD4 receptor. In this particular fusion, the V1V2 domains contain 179 amino acids.

On page 38, the paragraph at line 4:

5'-GCAGAACCAGAAG**GACGCC**AAGGTGGAGTTC-3' (SEQ ID NO. 13).

On page 38, the paragraph beginning on line 24:

Hybrid proteins of the type V1V2-HSA were generated by the following strategy: in a first step, plasmids pYG511 (Figure 28) and pYG374 (Figure 29) were respectively the source of the restriction fragments BglII-AhaII (fusion of the HSA prepro region and the V1V2 domains of the CD4 receptor) and AhaII-KpnI (in-frame fusion between mature HSA and the V1V2 domains of the CD4 receptor as exemplified in E.12.2.). Ligation of these fragments in a chloramphenicol resistant derivative of pBluescript II SK(+) vector (plasmid pSCBK(+), Stratagene) generates plasmid pYG537 (Figure 31). This plasmid contains a HindIII fragment coding for the hybrid bivalent molecule CD4-HSA-CD4 fused in-frame with the signal peptide of HSA as exemplified in E.11.2. Plasmid pYG547 which contains a HindIII fragment coding for the hybrid protein V1V2-HSA fused in-frame with the prepro region of HSA, was then derived by substitution of the PstI-KpnI fragment of pYG537 by the PstI-KpnI fragment from plasmid pYG371. The HindIII fragment carried by plasmid pYG547 can then be expressed under control of a functional yeast promoter cloned in a vector that replicates, for example, in yeasts of the genus Kluyveromyces. One example is the expression plasmid pYG560 whose structure and restriction map are shown in Figure 32. Vector pYG105 used in this

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particular example corresponds to plasmid pKan707 whose HindIII site has been destroyed by site-directed mutagenesis (oligodeoxynucleotide Sq1053, 5'-GAAATGCATAAGCTCTTGCCATTCTCACCG-3') (SEQ ID NO. 14) and whose SalI-SacI fragment coding for the URA3 gene has been replaced by a SalI-SacI fragment carrying a cassette made up of a promoter, a terminator, and a unique HindIII site.

On page 39, the paragraph beginning on line 30:

5'-ACTGCCAACTCTGACACC**TAAAAGCTT**GGATCCCACCTGCTTCAGGGGC AG-3' (SEQ ID NO. 15)

On page 41, the paragraph beginning on line 6:

5'-GGTAGGTCGTGTGGACGCCAGATCTTTGGAAAGAATTGCCCGTCTGGAA G-3' (SEQ ID NO. 16)

5'-CTGCAGGTTAGGCGTCGCCAACCAGTTGCTTCAGCTGTGC-3' (SEQ ID NO. 17)

On page 41, the paragraph beginning on line 28:

Proteins secreted by strain MW98-8C transformed by expression plasmids for HSA-CD4 hybrid proteins in which the CD4 moiety is carried on the MstII-HindIII fragment in the natural MstII site of HSA (plasmid pYG308B for example), were analyzed. Figure 35 demonstrates the presence of at least two cleavage products comigrating with the albumin standard (panel 2), which have a mature HSA N-terminal sequence, and which are not detectabe using polyclonal antibodies directed against

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human CD4 (panels 2 and 3). It is shown that these cleavage products are generated during transit through the yeast secretory pathway, probably by the KEX1 enzyme of K. lactis (or another protease with a specificity analogous to the endoprotease YAP3 of S. cerevisiae whose gene has been cloned and sequenced (Egel-Mitani M. et al. Yeast 6 (1990) 127-137). Therefore, the peptide environment of the hinge region between the HSA and CD4 moieties was modified, notably by fusion of the CD4 molecule (or one of its variants capable of binding the gp120 protein of HIV-1) to HSA N-terminal regions of varying length, according to the following strategy: plasmid pYG400 is an intermediate plasmid carrying the prepro-HSA gene, optimized with respect to the nucleotide sequence upstream of the ATG codon, on a HindIII fragment. This plasmid was linearized at its unique MstII site and partially digested by Bal31 exonuclease. After inactivation of this enzyme, the reaction mixture was treated with the Klenow fragment of E. coli DNA polymerase I and then subjected to ligation in the presence of an equimolar mixture of oligodeoxynucleotides Sq1462 (5'-GATCCCCTAAGG-3') (SEQ ID NO. 18) and Sq1463 (5'-CCTTAGGG-3') which together form a synthetic adaptor containing a MstII site preceding a BamHI site. After ligation, the reaction mixture was digested with HindIII and BamHI and fragments between 0.7 and 2.0 kb in size were separated by electroelution and cloned into an M13 mp19 vector cut by the same enzymes. 10^6 lytic plaques were thus obtained of which approximately one-third gave a blue color in the presence of IPTG and XGAL. Phage clones which remained blue were then sequenced, and in most cases contained an in-frame fusion between the HSA N-terminal moiety and β-galactosidase. These composite genes therefore contain HindIII-MstII fragments carrying sections of the N-terminal of HSA; Figure 36 shows several

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examples among the C-terminal two-thirds of HSA. These fragments were then ligated with a MstII-HindIII fragment corresponding to the CD4 moiety (for example the V1V2 domains of Figure 2, or the V1 domain alone), which generates HindIII fragments coding for hybrid proteins of the type HSA-CD4 in which the HSA moiety is of varying length. These restriction fragments were then cloned in the proper orientation into an expression cassette carrying a yeast promoter and terminator, and the assembly was introduced into yeasts. After growth of the culture, the hybrid proteins HSA-CD4 can be obtained in the culture medium; certain of these hybrids have an increased resistance to proteolytic cleavage in the hinge region (Figure 35).

On page 43, the paragraph beginning on line 20:

5'-GTGCTGGGCAAA**CAA**GGGGATACAG-3' (SEQ ID NO. 19)

5'-GGCTTAAAG**CAA**GTGGTGCTG-3' (SEQ ID NO. 20)

On page 44, the paragraph beginning on line 5:

5'-TGCTTTGCCGAGGAGGGTAAGGAAGACGCTAAGGGTAAGTCTGAAGAA

GAAGCCTTAGGCTTAAAGAAA-3' (SEQ ID NO. 21).

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